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Ian S. Goldlust Date

The Design of Theophylline Sensing Riboswitches in *Agrobacterium tumefaciens* and Their Application in Reprogramming the VirA/VirG Two-Component System

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An abstract of
A thesis submitted to the Faculty of the Graduate School of Emory University
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Abstract

The Design of Theophylline Sensing Riboswitches in *Agrobacterium tumefaciens* and Their Application in Reprogramming the VirA/VirG Two-Component System

By Ian Goldlust

Bacteria have evolved several mechanisms to sense and respond to chemical stimuli. One such mechanism, the riboswitch, is a *cis*-acting regulatory RNA that can bind metabolites and subsequently control gene expression and modify an organism's behavior.

Chapter one starts with an overview of synthetic and natural riboswitches and their mechanism of action. Chapter two presents a strategy to design and optimize synthetic theophylline-sensing riboswitches for the α-proteobacteria *Agrobacterium tumefaciens*—starting from weakly functioning *Escherichia coli*-optimized riboswitches, and moving towards the discovery a 19-fold riboswitch. Chapter three discusses the potential for increasing riboswitch function through the incorporation of an epsilon (ε) enhancer sequence into the expression platform. Finally, Chapter four begins by outlining a strategy to reprogram the VirA/VirG, two-component system of *A. tumefaciens* so that it responds only to the small molecule theophylline and ends by discussing a strategy to transport this system effectively into *E. coli*.

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I would also like to thank my family. They know that I would not be here today if not for them, literally and figuratively. It has been a difficult few years for us, and if I did not have them to fall back on and inspire me, I would not have made it this far.

I have got to thank all of my friends in Cleveland and Atlanta. It has been nearly six years since I have lived in Cleveland but every time I come home, it still feels the

same. I am confident that in six more years, I will be saying the same about the friends that I made in Atlanta.

Thanks to all of you.

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CHAPTER 1 – Chemical Sensing Through Small-Molecule RNA Interactions

1.1 Introduction

An organism's ability to detect metabolites in its environment and respond accordingly is critical for survival and overall fitness. Organisms have evolved several methods for genetic regulation based on the presence or absence of particular chemical signals. This kind of regulation usually relies on small molecule-protein interactions. The *lac* repressor is perhaps the most well-known example of a metabolite-sensing method of gene regulation. This system—characterized in *Escherichia coli*—represses gene expression by binding the *lac* repressor to the *lac* operator in the absence of chemical signals and preventing transcription (Jacob, 1961). When no lactose is present inside the cell, there is no need to waste energy producing the machinery necessary for its metabolism, and the *lac* repressor prevents transcription (Stoebel, 2008). When the concentration of lactose increases, however, allolactose (a lactose metabolite) binds to the *lac* repressor. This causes the repressor to dissociate from the *lac* operator, leading to the upregulation of the *lac* genes.

Although protein-based methods of gene regulation are more frequently discussed in the literature, other mechanisms do exist. Over the past decade, several RNA-based methods of gene regulation have been identified (Barrick, 2007). Metabolite-sensing RNAs, termed riboswitches, are most commonly found in the 5´ untranslated region (UTR) of a variety of mRNAs that regulate metabolic pathways (Nahvi, 2002; Winkler, 2002). The 12 identified classes of naturally-occurring riboswitches recognize a variety of metabolites, from nucleotides, to vitamins, to amino acids (Barrick, 2007). Most

commonly, riboswitches are found in the genomes of bacteria, specifically Eubacteria, but the discovery of riboswitches in eukaryotic genomes implies that they are more common in nature than previously thought (Sudarsan, 2003). Most of the naturally-occurring riboswitches have been discovered by using computational software that predicts conserved secondary structures and identifies conserved primary sequences in the UTRs of metabolic genes. The size and complexity of eukaryotic genomes makes it difficult to identify riboswitches using this method, but not impossible. The discovery of conserved riboswitch motifs in the UTRs of plant and fungi genomes have led some to suggest that riboswitches predate protein-based methods of genetic control—that they are relics of an ancient RNA-based world in which metabolism was controlled entirely by RNA (Sudarson, 2003).

The thiamine pyrophosphate (TPP) binding RNA supports this theory. Consensus sequences to the TPP metabolite binding domain have been identified in both Gram-positive and Gram-negative species of bacteria, plants, and fungi (Sudarson, 2003). In both prokaryotes and eukaryotes, TPP binding domains are located in the UTRs of genes that are involved in the thiamine biosynthesis pathway (Sudarson, 2003). Interestingly, TPP riboswitches have been found in both the 5´ and the 3´ UTRs of genes related to this metabolic pathway, leading to several theories regarding their function (Sudarson, 2003). In *E. coli*, the TPP riboswitch is located in the 5´ UTR and controls the translation of the gene *thiM*, a hydoxyethylthiazole kinase, by obscuring the Shine-Dalgarno sequence (RBS), which is necessary for translation, in the presence of TPP (Winkler, 2002). In *Bacillus subtilis*, the TPP riboswitch functions at the transcriptional level controlling the entire thiamine biosynthesis operon by forming a transcriptional terminator in the 5´ UTR

upon TPP binding (Mandal, 2003). In *Fusarium oxysporum*, a fungus, the TPP riboswitches location in the 5´ UTR between a 5´ and a 3´ splice site has led some to suggest that it functions by controlling the splicing of pre-mRNAs (Sudarson, 2003). Finally, in the plant species *Poa secunda* (bluegrass), the TPP riboswitch, which is found in the 3´ UTR, immediately adjacent to the polyA tail, have led some to suggest that it functions by regulating mRNA processing or perhaps by affecting RNA stability (Sudarson, 2003). The variety of mechanisms by which riboswitches can regulate gene expression in conjunction with their ubiquity in nature indicates that they developed over millions of years of evolution. This may lead some to believe that riboswitches are inherently complex, but this is not the case.

Riboswitches are comprised of two domains: (1) a metabolite sensing domain, known as an aptamer, and (2) an expression platform, responsible for conferring metabolite binding to an increase or decrease in downstream gene activation (Nudler, 2006). The aptamer domain is highly specific and can distinguish between molecules that differ in as little as one methyl group. Upon ligand binding, conformational changes control downstream elements that regulate transcription or translation (Nudler, 2006). Most of the riboswitches that our laboratory selects for are translational riboswitches that activate gene expression in the presence of a small molecule by freeing the RBS upon ligand binding (Desai, 2004; Lynch, 2008; Topp, 2008).

There are some straightforward methods that exist for selecting aptamers that can tightly and specifically bind a variety of ligands, from small functionalized molecules to large proteins. It has previously been demonstrated that aptamers can be selected from large pools of random RNA sequences (Winkler, 2004). Using a process called SELEX

(**S**ystematic **E**volution of **L**igands by **EX**ponential enrichment), Tuerk and Gold (1990) reported that they could isolate aptamers that bind proteins. In 1994, an aptamer that binds the xanthine alkaloid theophylline, the mTCT8-4 aptamer, was discovered (K_D = 320 nM) (Jenison, 1994). Despite differing by only one methyl group, the theophylline aptamer binds caffeine 10,000-fold less tightly than theophylline (Jenison, 1994).

Selecting a metabolite binding RNA may be straightforward, but taking an aptamer and making a functioning riboswitch is much more challenging. By employing high-throughput screens and selections we have shown that it is possible to select for riboswitches *in vivo*. Briefly, we have designed selection schemes where randomized libraries of riboswitch controlled chemotactic, enzymatic, or fluorescent genes can be used to identify new or improve already discovered riboswitches (Desai, 2004; Lynch, 2007; Topp, 2008). From these experiments we have gained crucial knowledge into the mechanism of action of synthetic and natural riboswitches and can now design theophylline riboswitches using only the aid of RNA folding software or low-throughput, easily manageable screens.

We have also demonstrated that our theophylline riboswitches are capable of regulating complex signaling pathways and have designed numerous methods for optimizing and tailoring riboswitches for specific applications (Topp, 2007). The ability to control bacterial behavior using small molecules that are of interest to us has profound biochemical and biotechnological implications. Recent efforts in our lab have focused on reprogramming bacteria so that they are capable of seeking out and degrading the herbicide atrazine, one of the most commonly used herbicides in America (Joy Sinha, unpublished data). By controlling the chemotaxis pathway with riboswitches, these “seek and

destroy" bacteria are only motile in the presence of atrazine, and remain stationary if no atrazine is present. Chemical-induced systems, like this one, may be used in the future to clean up a variety of environmental hazards, like oil spills or pollution in water supplies.

CHAPTER 2 – The Design and Optimization of Transportable Synthetic Riboswitches in *Agrobacterium tumefaciens*

2.1 Introduction

The possibility of using synthetic riboswitches to control gene expression in *Agrobacterium tumefaciens* has not been explored. The discovery of a naturally occurring S-adenosylmethionine (SAM) riboswitch in *A. tumefaciens* suggests that controlling gene expression in this organism through small molecule-RNA interactions is, in fact, possible (Corbino, 2005). Attempts to use other artificial methods of gene regulation, such as the *lac* repressor, to control complex signal transduction pathways in *A. tumefaciens* have failed to restore the absolute levels of expression observed in the wild-type cells (Lohrke, 2001). A need therefore exists for novel methods of tunable gene regulation in *A. tumefaciens* to facilitate the study of these complex pathways.

We have shown that it is possible to regulate the complex chemotaxis pathway in *E. coli* using synthetic riboswitches. Chemotaxis in *E. coli* is dependent on six chemotaxis proteins (Che A, B, R, W, Y, and Z) that work in concert with each other in order to confer ligand binding to a chemoreceptor to a change in rotational direction of the flagellar motor (Bren, 2000). If CheY is phosphorylated, the flagellar motor rotates clockwise and cells exclusively tumble in place and are non-motile (Wolfe, 1987). When CheZ removes the phosphate group from CheY, however, the flagellar motor switches direction, rotates counterclockwise, and the cells can swim smoothly on semi-solid agar (Zhao, 2002). Placing *cheZ* under the control of a riboswitch couples CheY dephosphorylation, and subsequently chemotaxis, to extracellular concentrations of theophylline.

The ability to control complex pathways, like the chemotaxis pathway, shows the utility of riboswitches over protein-based methods of gene regulation. If CheZ expression is too high, the bacteria become embedded in the media and are non-motile on semi-solid agar (Wolfe, 1989). Thus, the regulation of this pathway relies on the ability to tune synthetic riboswitches for specific applications. Since the *lac* repressor is promoter-dependent, the absolute levels of gene expression are hard to modulate. Riboswitches cannot only be modulated by selecting different promoters, but can be tuned by RBS modification. Ultimately, complementarity between the RBS and the 3´ end of the 16S rRNA determines the strength of the RBS, where increasing complementarity confers an increase in gene expression (Gold, 1988). Ligand dependent chemotaxis requires the coupling of a strong promoter (PTAC) and a weak RBS (Topp, 2007). If theophylline riboswitches that function in *A. tumefaciens* could be identified, then it may be possible to use them to control the signaling pathways that cannot be adequately regulated by other protein-based methods.

2.2 Results and Discussion

2.2.1 Testing Previously Identified *E. coli* Riboswitches in *A. tumefaciens*

Initial screens of *E. coli* optimized riboswitches in *A. tumefaciens* failed to produce significant gene activation in the presence of theophylline. Riboswitch 8.1, which shows 22-fold activation in *E. coli* (MDS42) failed to activate gene expression in *A. tumefaciens* upon the addition of theophylline. The low levels of expression in the presence and absence of ligand implies that riboswitch 8.1's extremely weak, two-base ribosome binding site, may have been unrecognizable by the 16S rRNA of *A. tumefaciens*.

Riboswitch 8.2, which shows 33-fold activation in *E. coli* (TOP10) and also contains a weak RBS, exhibited only 4-fold activation at 1 mM theophylline (Lynch, 2007). It was hypothesized that *A. tumefaciens* may require a much stronger RBS in order to restore the induction ratios seen in *E. coli*. Evidence in the literature supported this theory (Golshani, 2000a; Golshani, 2000b). It has been shown, that translation can be initiated in *E. coli* even when no RBS is present, but similar experiments have shown that this is not the case for *A. tumefaciens* (Golshani, 2000b). To verify this hypothesis, it was necessary to test constructs with stronger RBSs.

2.2.2 Testing Previously Identified *A. baylyi* Riboswitches in *A. tumefaciens*

The purine rich RBS of riboswitch "420" that was isolated in a *de novo* screen in *A. baylyi* contains a relatively strong, seven base RBS. In support of our previous hypothesis, this riboswitch functioned better in *A. tumefaciens*, exhibiting 7-fold activation at 1 mM theophylline. Guided by this information, it was hypothesized that the 16S rRNA of *A. tumefaciens* requires a very strong interaction between the ribosome and the mRNA. It is known that Gram-positive bacteria also require such strong interactions and examination of the 3′ end of the 16S rRNA of the *A. tumefaciens* and *B. subtilis* genes showed that the ideal

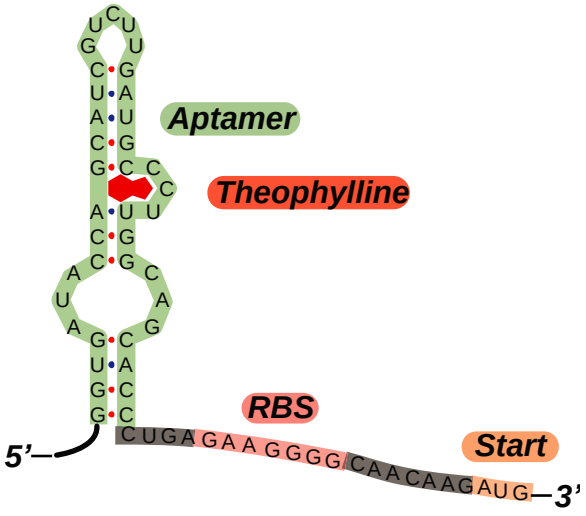

Figure 2.1 - Predicted Secondary Structure for the *A. baylyi*-Optimized Riboswitch "420". Predicted fold for the *A.baylyi*-optimized synthetic riboswitch in the presence of theophylline. The aptamer is shaded in green and the Shine-Dalgarno (RBS) sequence is shown in pink.

RBSs were nearly identical. Previous work in our lab involved screening for riboswitches in *B. subtilis* and these low-throughput screens were repeated in *A. tumefaciens.*

2.2.3 Low-Throughput Screening and Testing of Previously Identified Gram-Positive Riboswitches in *A. tumefaciens*

Gram-positive bacteria require a strong interaction between the 16S rRNA and target mRNA. Accordingly, two 256-member libraries were constructed with semi-ideal RBSs for Gram-positive species and two random bases flanking the RBS on either side. Previous high-throughput screens had focused primarily on the identification of ideal RBSs but such experiments were time consuming and costly. Since the ideal RBS for Gram-positive bacteria was already known, we were able to create a relatively small library that only screened for interactions between the region surrounding the RBS and the aptamer. The ability to suppress gene expression in the absence of ligand depends of the extent of the mRNA folding when the ligand is not bound. As a result, changing the RBS can have unforeseen effects on the activity of a riboswitch. A nearly ideal RBS was chosen, "AGGGGGT," because it showed significant homology to the anti-Shine-Dalgarno sequence for Gram-positive bacteria and it interacted strongly with the aptamer in the absence of ligand, allowing us to focus our libraries on optimizing the surrounding bases. Library 425 contained the complete "CAACAAG" motif preceding the start codon that is necessary for riboswitch function. mFold predictions indicated that the first base in the "CAACAAG" region could be optimized and, as a result, a second library (423) was created with the first cytosine deleted (Shana Topp, unpublished).

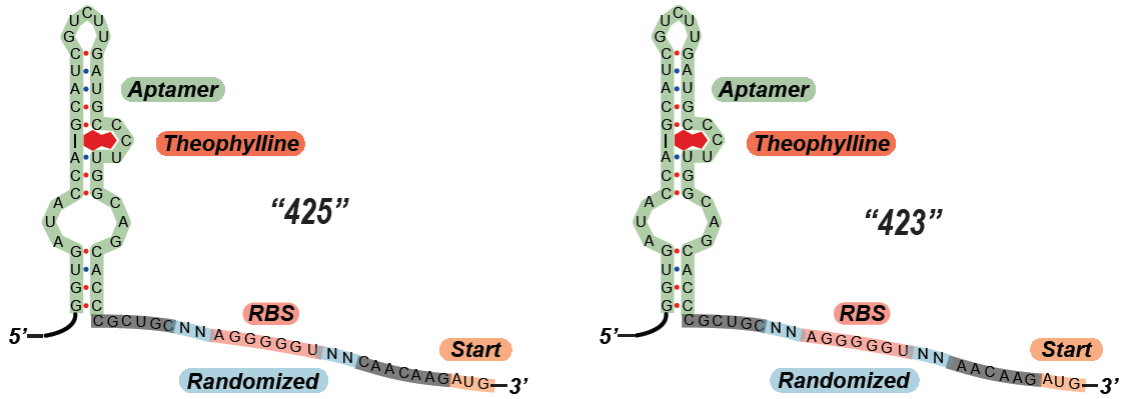

Figure 2.2 – Low-throughput Libraries to Identify Riboswitches for *B. subtilis*. Two 256-member libraries were designed for identifying synthetic riboswitches that function in *B. subtilis*. A semi-ideal RBS is flanked on either side by two random bases. Library "425" (left) contains the complete constant region, shown to pair best with the aptamer in the absence of ligand, following the RBS (shown in grey). Library "423" (right) contains a cytosine deletion in the first base of the constant region following the RBS (shown in grey). The aptamer is shaded in green and the Shine-Dalgarno (RBS) sequence is shown in pink.

96 members of each library were screened in *A. tumefaciens* and 47 members of library 425 were screened in the *E. coli*. Several clones were identified and tested for activity in *E. coli*, *B. subtilis*, *A. baylyi* and *A. tumefaciens*. One clone in particular, D2, showed 20-fold activation in *E. coli*, 65-fold activation in *B. subtilis*, 32-fold activation in *A. baylyi*, and 13-fold activation in *A. tumefaciens*. This suggested that it was in fact possible to design and transport riboswitches from organism to organism with no modification of promoter, vector, or RBS.

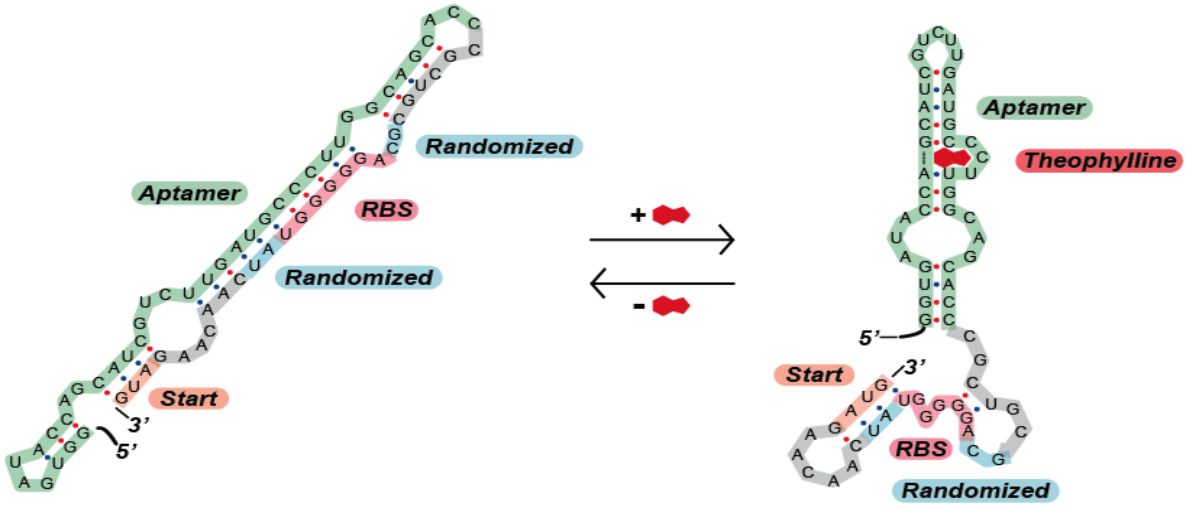

Figure 2.3 - Predicted Secondary Structure for the *B. subtilis*-Optimized Riboswitch "D2". Predicted fold for the *B. subtilis*-optimized synthetic riboswitch, D2, in the presence of theophylline. The aptamer is shaded in green, the Shine-Dalgarno (RBS) sequence is shown in pink, and the randomized region is highlighted in blue.

2.2.4 Towards Identifying Better Riboswitches in *A. tumefaciens*

Data to this point suggested that synthetic riboswitches performed worse in *A. tumefaciens* than the other organisms that were tested. To reconcile this difference, additional library screens specifically targeted at making better *A. tumefaciens* riboswitches were carried out by screening a library of RBSs. While screening for off-riboswitches, riboswitches that turn off gene expression in the presence of a small molecule, a library was designed with 12 rando-

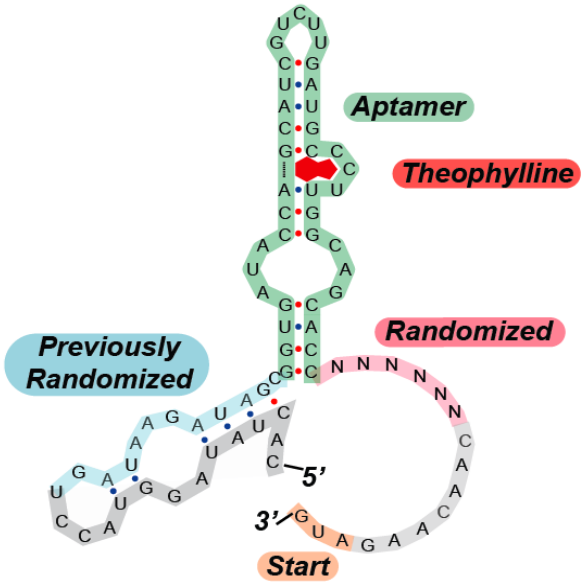

Figure 2.4 – Semi-Rational "Foot Library" for Identifying Riboswitches to Function in *A. tumefaciens*. A 4096-member library was designed for identifying synthetic riboswitches that function in *A. tumefaciens*. The ideal Shine-Dalgarno sequence (RBS) is screened for in the randomized region. The aptamer is shaded in green, the randomized region is shown in pink, and the previously randomized region is highlighted in blue

mized bases directly 5′ to the aptamer stem and a weak four-base RBS 3′ to the aptamer (Shana Topp, unpublished). Surprisingly when initial screens of this library were carried out in *E. coli*, it was noticed that a significant number of the clones showed strong activity in the presence of theophylline. The clone that showed the highest activation (10-fold) was sequenced and its randomized region is shown in blue in Figure 2.4.

Since that library showed such high theophylline-dependent activation, the six bases that were directly 3′ to the aptamer stem were randomized in order to screen for ideal RBSs. In addition to this library, termed the "foot library," a semi-rationally designed "single clone" was designed using mFold predictions and ensuring that the riboswitch contained a strong RBS that interacted tightly with the aptamer in the absence of ligand.

96 colonies were screened in *A. tumefaciens* and three clones were isolated for additional testing; the clone that showed the highest activation was the semi-rationally designed single clone. When assayed, the single clone showed 19-fold activation in the presence of 1 mM theophylline. It is worth noting, however, that the library was cloned from a plasmid containing the single clone sequence and was most likely contaminated with a higher percentage of single clone copies than any other random sequence. As a result, this screen should be repeated to attain more complete library coverage.

The single clone riboswitch was tested in *E. coli* and showed activation in the presence of theophylline, supporting the theory that riboswitches designed in one organism can function well in other organisms. The single clone had a similar activation ratio as other switches that were previously identified but had significantly different absolute levels of gene activation. In *E. coli*, the single clone showed essentially no activation in

the absence of ligand (6 Miller units) and moderate activation in the presence (1000 Miller units). Previously our "best" riboswitch in *E. coli* was very leaky and therefore not well suited for applications that require extremely low gene activation in the absence of ligand. Thus, the single clone riboswitch may find utility in specific cases in which extremely low background is desirable.

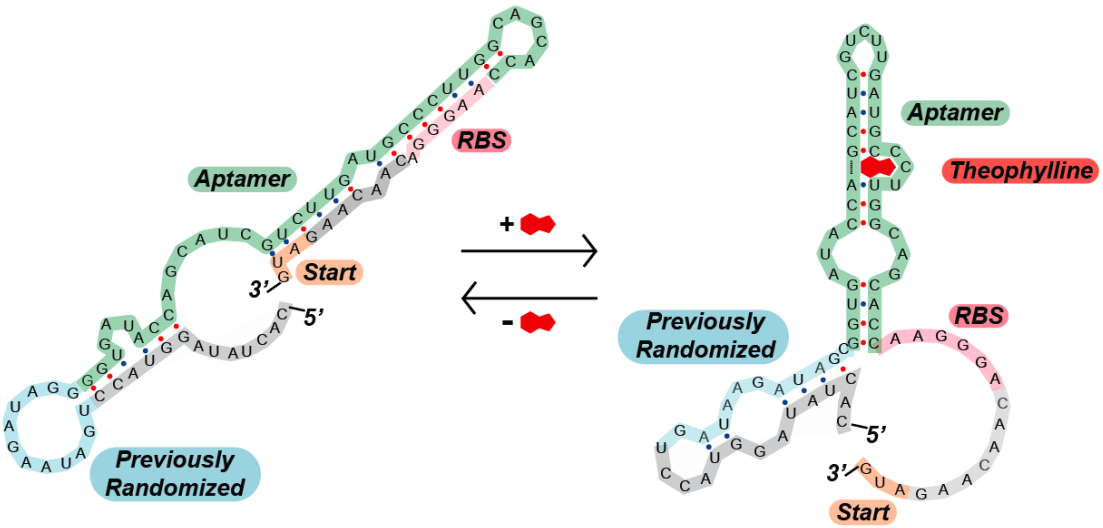

Figure 2.5 - Predicted Secondary Structure for Semi-Rationally Designed "Single Clone" Riboswitch. Predicted folds for the "Single Clone" synthetic riboswitch in the absence of ligand (left) and the presence of theophylline (right). The aptamer is shaded in green and the Shine-Dalgarno (RBS) sequence is shown in pink.

2.3 Conclusion

Initial attempts to design transportable synthetic riboswitches proved to be a difficult task. The accumulation of data from numerous high-throughput screens and experiments designed to elucidate the exact mechanism of action of a theophylline dependent riboswitch have made it possible for us to design riboswitches that perform well in a multitude of organisms. Several considerations, shuttle vector, promoter, and RBS, need to be taken into account when designing transportable riboswitches. Shuttling riboswitches

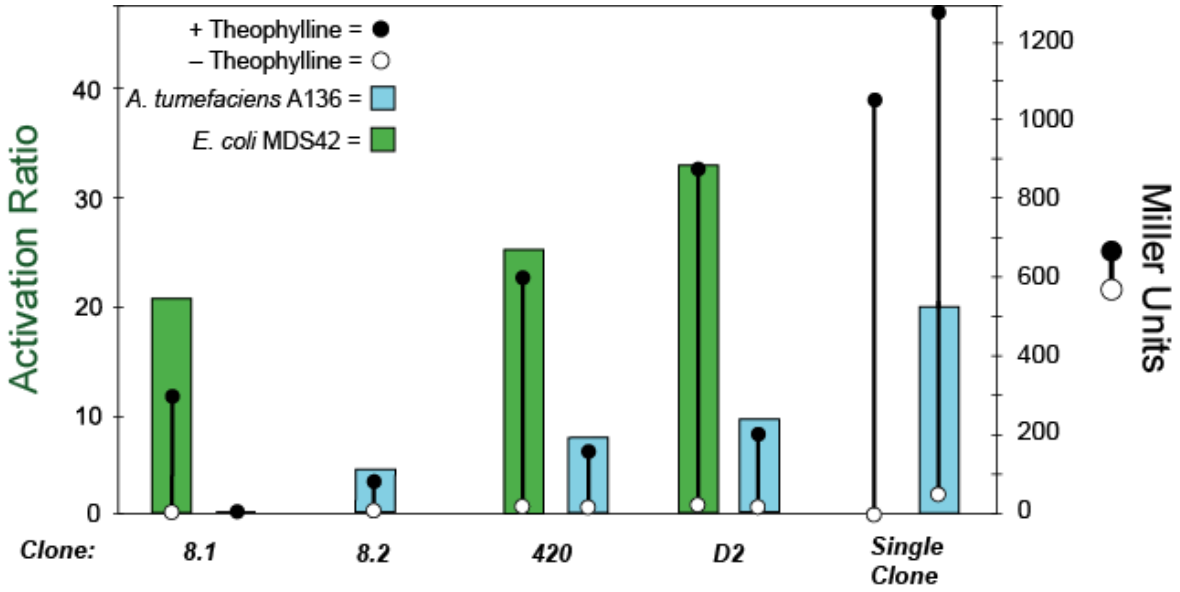

Figure 2.6 – Comparison of Riboswitch Function in *Agrobacterium tumefaciens* and *Escherichia coli*. Comparison of riboswitch function in *A. tumefaciens* and *E. coli* at 1 mM theophylline. Data for riboswitch 8.2 in *E. coli* MDS42 is not available. Activation ratio for the single clone riboswitch in *E. coli* cannot be determined because of the low levels of gene expression in the absence of ligand.

originally designed to function in *E. coli* to *A. baylyi* failed initially. The use of the *tac* promoter in *A. baylyi*, which has not previously been characterized in this organism, led to the hasty assumption that synthetic riboswitches may not be transportable. The initial shuttle vector, a pWH1266 derived vector, exhibited gene expression even in the absence o a promoter (Hunger, 1990). The most likely reason is that the vector contained an additional promoter sequence and was, therefore, not well suited for our current application. The recent acquisition of a broad-host-range shuttle vector, pBAV1K (Anton Bryksin), has aided attempts to design and transport switches from organism to organism with little or no modification. Once cloned into pBAV1K and tested again, the same switches that failed initially, showed modest activity (see figure 2.6).

Based on these results and the numerous experiments and high-throughput screens previously carried out, certain predictions about the function of *E. coli* riboswitches in *A. tumefaciens* can be made. We are confident that we can make semi-rational modifica-

tions to optimize these riboswitches for specific applications. By increasing the strength of the RBS it is possible to design switches that function well in *E. coli* and *A. tumefaciens* culminating in the discovery of a 19-fold riboswitch in *A. tumefaciens*. This evidence suggests that the ribosome of *A. tumefaciens* is less promiscuous than that of *E. coli* or other Gram-negative bacteria.

2.4 Experimental

General Considerations

Synthetic oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Culture media was purchased from END Bioscience. Theophylline and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were obtained from Sigma. Kanamycin was purchased from Fisher Scientific. DNA polymerase, restriction enzymes, and ligase were purchased from New England Biolabs (Ipswitch, MA). Plasmid manipulations were performed in *E. coli* MDS42 (Scarab Genomics), *A. tumefaciens* C58, and *A. tumefaciens* A136; *A. tumefaciens* strains were kind gifts of Dr. David Lynn. Plasmid, PCR products, and digestions were purified using kits purchased from Qiagen (Valencia, CA). All new constructs were verified by sequencing performed by MWG Biotech.

Transformation Protocols

A. tumefaciens strains C58 and A136 were transformed by the method described by Cangelosi et al (Cangelosi, 1991). Competent cells were grown for 18 hours in 5 mL LB at 28°C while shaking at 250 rpm. Once saturated, 10-100 μL was added to 50-150 mL of fresh LB and allowed to grow until the OD_{600} = 0.4. The cells were then pelleted

three times by centrifugation at 5000 *g*, washing twice with water and once with 10% glycerol. 50 μL were aliquoted and, either used fresh, or flash frozen in liquid N_2 and stored at -80°C for later use. Transformations were performed using an Eppendorf Electroporater 2510 (Westbury, NY) at 1800 volts. 1 μL of plasmid DNA was transformed and transformants were allowed to recover in 500 μL SOB for 4 hours. 10-100 μL of culture was then plated on solid LB media with the appropriate antibiotic.

Assays for β-galactosidase Activity

Library transformations were plated on solid LB agar supplemented with kanamycin (KAN) (50 μg/mL). Cells were grown for 18-24 hours or until they were large enough to pick by hand. 96-well titer plates (Costar) with 200 μL of LB media supplemented with KAN (50 μg/mL) were inoculated and left to grow while shaking (180 RPM) until saturated. Once saturated, four 96-well plates (two that contained 200 μL of LB with no small molecule, two that contained 200 μL of LB supplemented with 1mM theophylline) were inoculated with 2.5 μL of saturated culture. Plates were incubated for 5-8 hours to an OD_{600} of 0.1-0.2 as determined by a Biotek microplate reader; this corresponds to an OD_{600} value of approximately 0.3-0.5 with a 1 cm path length cuvette.

Assays for β-galactosidase activity were adapted from previously described methods (Lynch, 2007). Cultures were lysed by adding 20 μL of Pop Culture ® solution (Novagen) and were mixed by pipetting up and down three times. They were allowed to lyse at 28°C for 5 minutes. In fresh plates, 15 μL of lysed culture was added to 132.2 μL of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-

mercaptoethanol, pH 7.0). This was followed by the addition of 29 μL of ONPG (4 mg/mL in 100 mM NaH_2PO_4). ONPG was allowed to hydrolyze at 28°C until a yellow color developed (10-60 minutes). The reaction was quenched by addition of 75 μL of Na_2CO_3 (1 mM). The hydrolysis time was recorded and the OD_{420} was determined for each well.

Data Analysis

Miller Units were calculated according to Equation 1 and activation ratios were calculated according to Equation 2.

$$\text{Miller Units} = \frac{OD_{420} * 1000}{OD_{600} * \text{Volume Assayed (mL)} * \text{Hydrolysis Time (min)}}$$

Equation 1 - Miller Units Definition

$$\text{Activation Ratio} = \frac{\text{Miller Units with Theophylline}}{\text{Miller Units without Theophylline}}$$

Equation 2 - Activation Ratio Definition

The clones with the highest activation ratios were examined for aberrations in the data; clones that were overgrown, undergrown, had OD_{420} values <0.04, or showed dramatic differences between the two plates were eliminated from consideration. Clones that were selected were subcultured and assayed quantitatively according to the method described by Lynch et al (Lynch, 2007). Cells were grown in duplicate and assayed in duplicate. OD_{600} and OD_{420} values were taken in duplicate and averaged in order to minimize pipetting error.

Plasmid Construction

"420"

Primers ST420 and ST412 were used to amplify the riboswitch sequence and *lacZ* gene from pSKD11.2 (Desai, 2008). The resulting PCR product was digested with *Pst*I and *Xba*I. A Biobrick Accepting Vector (pBAV1K; gift from Anton Bryksin) was digested with *Spe*I and *Pst*I, and was dephosphorylated with CIP. The two DNA fragments were ligated, and the precipitated ligation was used to transform *E. coli* strain MDS42. This transformation yielded a strain harboring p420.

"Library 423"

Primers ST423 and SKD56 were used to amplify the *IS10* sequence from p420. Primers JPG007 and ST424 were used to amplify the 5′ UTR portion of p420. The full length PCR product was assembled by combining these two cassettes and amplifying them with primers JPG007 and SKD056. The resulting DNA was digested with *Hind*III and *Kpn*I. After digestion of p420 with *Hind*III and *Kpn*I, the vector was dephosphorylated with CIP. These DNA fragments were ligated, and the precipitated ligation was used to transform *E. coli* strain MDS42.

"Library 425"

Library 425 was constructed as indicated for "Library 423", except primer ST425 was used in place of ST423, and primer ST426 was used in place of ST424.

“8.1” and **“8.2”**

Primers JPG007 and SKD056 were used to amplify the riboswitch sequences and the *IS10* sequence from p8.1 or p8.2 (Lynch et al., 2007). Restriction digests and ligations were performed as described for “Library 423.”

“Single Clone”

Primers ST473 and SKD56 were used to amplify the *IS10* sequence from pST5565 (Shana Topp, Dissertation, Figure 4.2). Primers JPG007 and ST474 were used to amplify the 5′ UTR portion of pST5565. Digestions and ligations were performed as described for “Library 423.”

“Foot Library”

The foot library was constructed as indicated for “Single Clone”, except primer ST475 was used in place of ST473, and primer ST476 was used in place of ST474.

CHAPTER 3 – Using Epsilon (ε) Sequences to Enhance the Activation of Synthetic Riboswitches

3.1 Introduction

In vivo applications of riboswitches require precise tuning of gene expression. By changing the RBS or the promoter, we can reduce gene expression, but few strategies exist that are capable of increasing the activation ratio of a riboswitch without the involvement of complex screens or selections. Therefore, a need exists for the discovery of additional tools that can be used to enhance the activity of riboswitches *in vivo*.

The initiation of mRNA translation in *E. coli* relies on base pairing between a purine rich sequence, the RBS, and its complementary sequence at the 3´ end of the 16S rRNA (Shine, 1975; Gold, 1988). Different organisms contain different 16S rRNA sequences and subsequently have different anti-Shine-Dalgarno sequences. When the complementarity between an RBS sequence of a specific mRNA and the anti-Shine-Dalgarno of its corresponding rRNA is reduced, gene expression drops (Gold, 1988). Similarly, weak base pairing between a RBS sequence and the 16S rRNA confers a decrease in both activation ratio of a riboswitch and a decrease in the absolute levels of gene expression. However, weakening a RBS sequence can only be used to decrease activity; strengthening a RBS can yield greater protein expression, but most riboswitches found to date already contain ideal RBSs. A second means used to alter gene expression involves selecting different promoter sequences upstream of the aptamer domain (Topp, 2007). Although this can affect the absolute levels of gene expression, it has little affect on the activation ratio of a riboswitch.

One method of enhancing gene expression that has not been explored in the context of a riboswitch is the introduction of an ε sequence in the 5´ UTR. An ε enhancer sequence is a sequence that is complimentary to helical domain #17 of the 16S rRNA, nucleotides 458-467 in the *E. coli* rRNA and 414-422 in the *A. tumefaciens* rRNA. Its incorporation into the 5´ UTR can increase translational activity without promoter or RBS modification (Olin, 1989). In *E. coli* and *A. tumefaciens*, the presence of an ε enhancer sequence can increase CAT gene expression by nearly two-fold and three-fold respectively (Golshani, 2000a). Additionally, in *E. coli*, the ε sequence alone is capable of initiating translation in the absence of a RBS sequence (Golshani, 2000b).

Exactly how the ε sequence activates or enhances translation remains unclear. The presence of sequences complementary to helical loop number #17 in the 5´ region of some highly expressed *E. coli* genes suggests that enhancement is dependent on base pairing interactions (Golshani, 2004). However, other work has shown that the secondary structure of the mRNA and not the primary sequence dictates ε sequence function (O'Connor, 2001).

These previous reports lead one to hypothesize that incorporation of an ε sequence between the aptamer domain and RBS of a riboswitch may enhance the activation ratio of a riboswitch in two ways. First, the introduction of additional bases that can potentially interact with the aptamer domain in the off state could sequester both the ε sequence and the RBS, reducing gene expression in the absence of ligand. Second, when the ligand is bound, both the ε sequence and the RBS are free to interact with the ribosome, increasing gene expression in the presence of ligand. Both of these effects would lead to an increase in the activation ratio of a riboswitch.

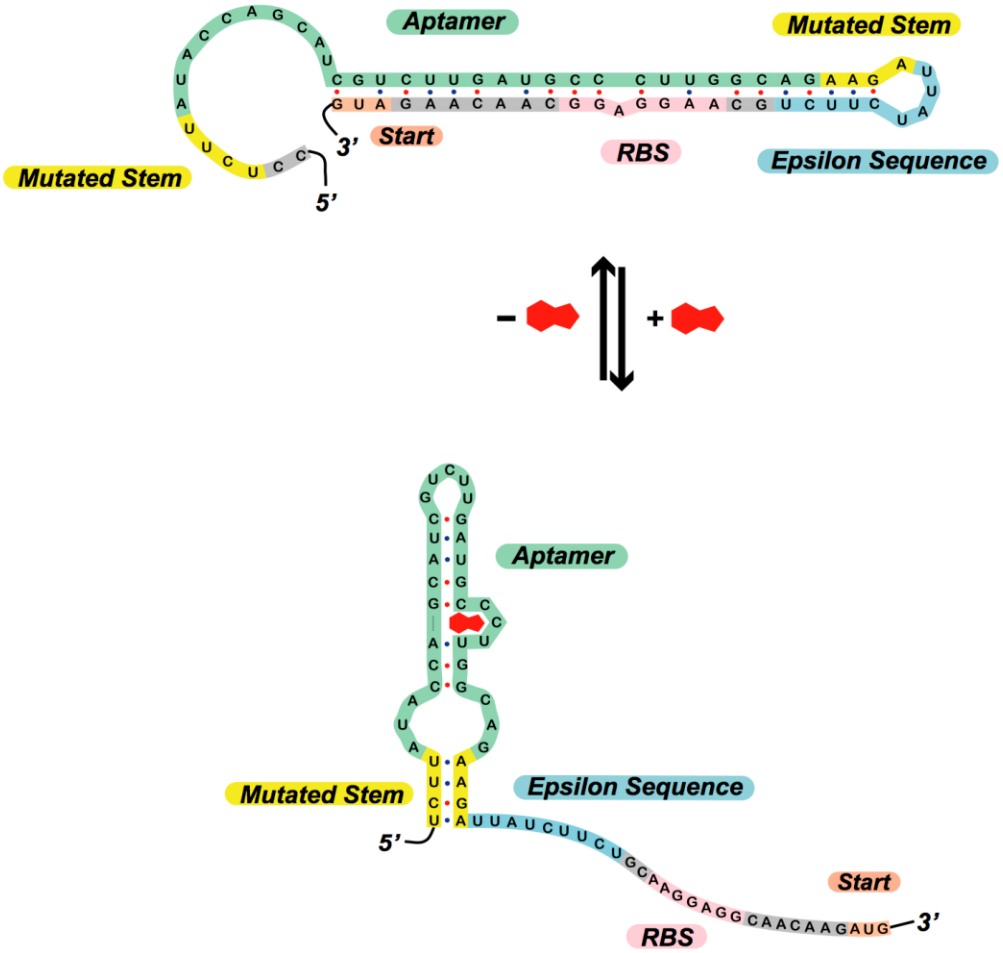

Figure 3.1 - Predicted Secondary Structure for the ε Enhanced Riboswitch. Predicted folds for the ε enhanced synthetic riboswitch in the absence of ligand (top) and the presence of theophylline (bottom). The aptamer is shaded in green, the mutated aptamer stem is shown in yellow, the ε sequence is highlighted in blue, and the Shine-Dalgarno (RBS) sequence is shown in pink.

3.2 Results and Discussion

Incorporation of an ε sequence homologous to the #17 helical loop domain of the 16S rRNA of *A. tumefaciens* resulted in a complete loss of riboswitch activity. When cloned upstream of *lacZ* and tested for β-galactosidase activity, no dose dependent gene activation was seen. As concentrations increased, the levels of activity remained low, implying that switching function had been lost and that, even in the presence of ligand,

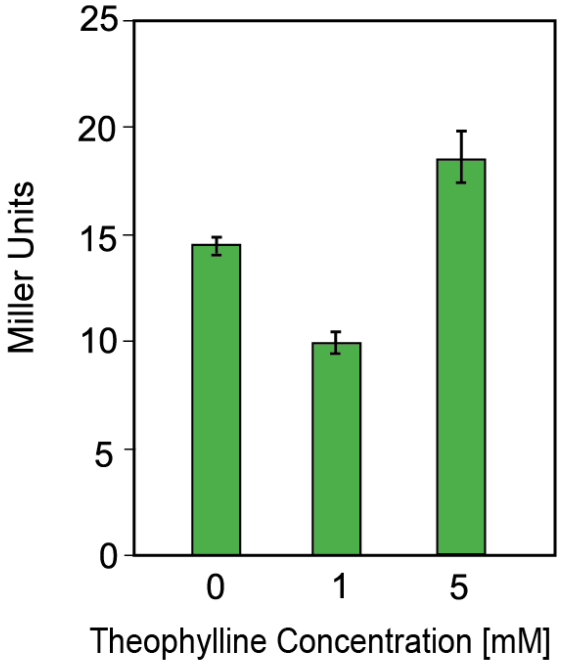

Figure 3.2 - β-galactosidase Assay for an ε Enhanced Riboswitch Shows No Dose Dependence. Results of a β-galactosidase assay of the ε enhanced riboswitch shows that no dose dependent gene activation occurs. Low expression in the presence of ligand implies that the Shine-Dalgarno sequence is obscured. This data suggests that ε enhancement relies on secondary structure interactions.

the RBS and the ε sequence were obscured. This could be the result of a number of contributing factors.

Others have reported that either an 8 or a 16-base spacing between the end of the ε sequence and the start codon is ideal (Golshani, 2000b). The 16-base spacing was chosen because of the length of the RBS and the necessity of the "CAACAAG" motif in the expression platform. Additional screening would be necessary in order to elucidate what the ideal spacing would be in the context of riboswitch. Additionally, m-Fold predictions of the secondary structure of the ε sequence when the ligand is bound did not resemble that of the #17 helical loop of the 16S rRNA. If ε enhancement relies on secondary structure interactions, and is not sequence specific, then no enhancement activity would have been possible.

3.3 Conclusions

The incorporation of an ε sequence into the expression platform of a riboswitch conferred a complete loss of riboswitch activity. Additional experiments would need to be carried out to order to determine whether or not it would be possible to see an effect on gene expression. High-throughput screening of libraries with varied spacing or prima-

ry sequence would be helpful in answering this question but were not practical for the purposes of this experiment. The scarcity of research pertaining to ε sequences in general makes troubleshooting problems difficult. The potential 2-fold increase in gene expression was not considered significant enough to pursue with additional experiments. Regardless, the data suggests that ε enhancement requires more than a complementary RNA sequence; this suggests that specific secondary structure in the presence of ligand must be maintained in order to see enhanced translation. The drastic conformational changes that take place as a result of ligand binding make it hard to predict the secondary structure of the 3´ end of a riboswitch even with the aid of RNA folding software.

If the function of ε enhancement depends solely on secondary structure interactions, ε enhancement would provide a powerful tool for designing riboswitches with different aptamers. The theophylline aptamer contains a pyrimidine rich region that pairs tightly with purine rich RBSs. Aptamers that bind other metabolites may lack pyrimidine rich stretches and would not for tight hairpins in the absence of ligand. The primary sequence of the RBS cannot be changed drastically without impacting riboswitch function. Since evidence suggests that ε enhancement is sequence independent, the primary sequence can be modified so that it is complementary to any aptamer in the absence of ligand, as long as secondary structure is maintained in the presence.

3.4 Experimental

Construction of Plasmids

A sequential PCR method was used to mutate the p420 into the ε containing construct. Primers pISG38 and SKD56 were used to amplify p420; Primers pISG39 and SKD56 were used to amplify that product; and primers pISG40 and SKD56 were used to amplify that product. The final construct was cut with *Hind*III and *Kpn*I and cloned into pBAV as previously described for "library 423."

CHAPTER 4 – Hijacking and Reprogramming the VirA/VirG Two-Component System of *A. tumefaciens*

4.1 Abstract

The use of a variety of organisms as heterologous systems for the expression and subsequent analysis of complex signal transduction pathways is currently limited by an inability to adequately transport these systems from host to host (Lohrke, 2001). Attempts to reproduce the virulence system of *A. tumefaciens* into *E. coli* have failed to reproduce significant upregulation of the genes necessary for infection to occur, the virulence (*vir*) genes (Lohrke, 2001). The inherent complexity of the VirA/VirG two-component signal transduction pathway makes its expression in *E. coli* possible, but difficult. Simplifying this system would not only allow for more effective transfer but would also provide an important tool for understanding and manipulating the mechanism of transfer DNA (T-DNA) transfer. The low levels of *vir* gene induction in *E. coli* have been used as evidence to suggest that other *A. tumefaciens* genes are involved in this pathway (Lorhke, 2001). The ability to reproduce the absolute levels of virulence gene induction seen in *A. tumefaciens* in *E. coli* would provide evidence to the contrary. To restore the activity of this system, the signal recognition machinery must be simplified so that the entire virulence system only responds to a single extracellular signal. In addition, the system must be made so that it is transportable from organism to organism.

4.2 Introduction

4.2.1 The Virulence System of *Agrobacterium tumefaciens*

A. tumefaciens is a soil dwelling, α-proteobacterium that is responsible, in nature, for infecting dicotyledons (flowering plants whose seeds have two embryonic leaves) with crown gall tumors (Heath, 1995). This is the only known example in nature of interkingdom DNA transfer and its pathogenesis and virulence phenotype is well studied in the literature (Jiang, 2003). These infection events usually occur at wound sites and are dependent on the expression of *vir* genes. Upregulation of the *vir* genes depends on a variety of metabolites, phenols, sugar, and/or a decrease in pH, conditions that often occur when plants are wounded (Zhu, 2000; Binns, 2002). The genes necessary for virulence are localized on an extrachromosomal, 200 kb plasmid termed the Ti plasmid.

The Ti plasmid contains three distinct regions; the T-DNA region, a region responsible for opine catabolism, and the *vir* region (Heath, 1995). When *A. tumefaciens* infects a plant host, the T-DNA region is clipped on either end and incorporated via a variety of *vir* proteins, into the chromosome of the host plant cell (Veluthambi, 1987). The T-DNA region contains the oncogenic genes necessary for crown gall tumor development (Akiyoshi, 1984). Biotechnology has exploited this phenomenon by replacing the oncogenic genes with other genes of interest. Also located within the T-DNA region are genes that code for enzymes that produce specialized amino acids, termed opines (Veluthambi, 1989). The incorporation of these genes into the host genome causes the host to produce opines, that *A. tumefaciens* can metabolize and use. The second region of the Ti plasmid, termed the opine catabolism region, codes for the genes necessary for *A. tumefaciens* to utilize the opines produced by the host cell (Veluthambi, 1989).

The virulence region of the Ti plasmid is comprised of six genes that are responsible for a broad range of tasks. Two of the virulence genes, *virA* and *virG*, are regulatory genes that code for a two-component sensor-transducer system (Mukhopadhyay, 2004). VirA and VirG are responsible for regulating not only the production of the oncogenic genes but are also responsible for regulating the machinery necessary for the transfer of T-DNA (Mukhopadhyay, 2004).

As reported by Gao et al. VirA is comprised of two membrane-spanning domains; a periplasmic domain and cytoplasmic domain, which is subdivided into a linker domain, a kinase domain, and a receiver domain (Gao, 2005). The periplasmic domain is responsible for monosaccharide recognition via an interaction with the chromosomal sugar binding protein, ChvE. The perception of sugars is synergistically linked with phenol recognition, which is mediated through the linker domain. Decreases in pH are sensed by the pH-inducible *virG* promoters, P1 and P2. Interestingly, replacement of the wild-type promoters does not confer complete pH insensitivity, which implies that pH-regulation is not limited to these promoters. The kinase portion of VirA is responsible for phosphorylating VirG in the presence of extracellular signals. In the absence of these signals, however, the phophate group is merely passed to the receiver domain of VirA itself.

VirG is a DNA binding element that, when phosphorylated, translocates to the Ti plasmid and upregulates genes that contain a specific recognition sequence, a "*vir* box," in their promoter. VirG itself is divided into two sections. The receiver domain of VirG is homologous to the receiver domain of VirA and is phosphorylated at a conserved aspartate side chain. The DNA binding domain is responsible for recognition of the "*vir*

box" and subsequent recruitment of the ribosome, which is necessary for the upregulation of the virulence genes.

4.3 Experimental Design

4.3.1 Controlling the Virulence Pathway Using the Single Clone Riboswitch

To In order to more effectively study this signal transduction pathway and infection event this complex system must be simplified. *In vivo* maximum *vir* gene induction occurs when all three extracellular signals are present; as stated before, some of these signals are synergistically linked to one another. If it were possible to bypass VirA, so that maximum gene induction would occur as the result of just a single extracellular signal, then the system would be easier to study and easier to transport. To achieve this, the signal recognition domains of VirA must be ablated. If only the kinase domain of VirA is expressed with constitutively expressed VirG, then the upregulation of *vir* genes occurs in absence of any extra cellular signals. We can use this to our advantage by coupling *virG* expression to riboswitch activity. Controlling *virG* with the single clone riboswitch discussed in Chapter 2 would create a virulence system that responds only to theophylline. VirG would be produced dose-dependently in the presence of varying concentrations of theophylline. Any VirG produced would immediately be phosphorylated by the kinase domain of VirA and would upregulate any genes under the control of a *vir* promoter. By fusing a *vir* promoter, specifically the *virB* promoter (*virBp*), to lacZ (*virBp::lacZ*) we would be able to monitor the effect of theophylline on the VirA/VirG system.

To facilitate the transport of the virulence system into *E. coli*, the 89 C-terminal residues of the α-subunit of *A. tumefaciens'* RNA polymerase, the RpoA fragment, must be expressed along with the 247 N-terminal residues of *E. coli*'s RNA polymerase (Lorhke, 1999). The wild-type *E. coli* RNA polymerase is incapable of recognizing and interacting with VirG, which is essential for *vir* gene upregulation (Lorhke, 1999). The RpoA hybrid will be constitutively expressed along with the kinase portion of VirA and theophylline dependent VirG in order to create a system that will function in *E. coli*.

4.3.2 Additional Library Screens to Isolate Optimized Riboswitches for Controlling Virulence

If the single clone construct fails to show significant upregulation of *vir* genes, a randomized library will be screened in front of *virG* and functional riboswitches will be selected based on *virBp::lacZ* activity. A similar strategy was employed to optimize the *cheZ* riboswitches discussed in Chapter 2. Screening a library in front of *virG* instead of *lacZ* would screen for virulence activation as opposed to riboswitch function. Plating the library without theophylline on bromo-chloro-indolyl-galactopyranoside (X-gal), a substrate that when hydrolyzed by β-galactoside produces a blue color, will screen for clones that show no virulence activity in the absence of ligand. The whitest colonies will be screened as described in section 2.4. Once identified these riboswitches can be cloned in front of *lacZ* and assayed individually in order to identify their specific properties. This screen can also be repeated in *E. coli* to optimize this system for that organism.

List of Primers Used

pISG38
5´-AAGATTATCTTCTGCAAGGAGGCAACAAGATGTGCGAACTCGAACTCG-3´

pISG39
5´-GCATCGTCTTGATGCCCTTGGCAGAAGATTATCTTCTGCAAGGAGGCA
ACAAGATGTGC-3´

pISG40
5´-CGACTCACTATAGGTACCUCUUATACCAGCATCGTCTTGATGCCCTTGGC-
3´

ST412
5´-GTCGCCATCGATCGGGCCCTGAGGCCTGCAGCGGCCGCTACTAGTATT
ATTTTTGACACCAGACCAACTGGTAATGG-3´

ST420
5´-GGAATTCGCGGCCGCTTCTAGAGATACGACTCACTATAGGTACCGGTG
ATACC-3´

ST423
5´-CTTGGCAGCACCCGCTGCNNAGGGGGTNNCAACAAGATGTGCGAACT
CGATATTTTACAC -3´

ST424
5´-GTGTAAAATATCGAGTTCGCACATCTTGTTGNNACCCCCTNNGCAGCG
GGTGCTGCCAAG-3´

ST425
5´-CCTTGGCAGCACCCGCTGCNNAGGGGGTNNAACAAGATGTGCGAACT
CGATATTTTACAC-3´

ST426
5´-GTGTAAAATATCGAGTTCGCACATCTTGTTNNACCCCCTNNGCAGCGG
GTGCTGCCAAGG-3´

ST473
5´-GGCAGCACCAAGGGACAACAAGATGTGC-3´

ST474
5´-GCACATCTTGTTGTCCCTTGGTGCTGCC-3´

ST475
5’-CCTTGGCAGCACCNNNNNNCAACAAGATGTGCG-3´

ST476
5´-CGCACATCTTGTTGNNNNNNGGTGCTGCCAAGG-3´

JPG007
5´- GCGATTAAGTTGGGTAACGCCAGGG-3´

SKD056
5´-CGACGGGATCGATCCCCCC-3´

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